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Validation of Red Cell Sodium-Lithium Countertransport Measurement – Influence of Different Loading Conditions

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Summary: Increased sodium-lithium countertransport in erythrocytes from patients with long-standing type I (insulin-dependent) diabetes mellitus has been considered as an early marker of nephropathy. Since the activity and kinetics of the sodium-lithium countertransport may critically depend on loading conditions, this study was aimed at determining sodium-lithium countertransport activity, *Michaelis* constant K_m and maximum velocity V_{max} in erythrocytes loaded in two different Li^+ solutions. Sodium-lithium countertransport activity was determined in erythrocytes in 8 healthy control subjects after loading with 150 mmol/l LiCl compared with those loaded with 150 mmol/l $LiHCO_3$. Sodium-lithium countertransport activity was similar for both loading procedures, although the erythrocyte lithium content did significantly differ (mean ± SEM, 7.0 ± 0.5 for LiCl and 8.9 ± 0.5 mmol/l of cells for 150 mmol/l $LiHCO_3$). There were no significant changes in the K_m and V_{max}. Increase of osmolality in efflux media containing 200 and 250 mmol/l NaCl resulted in a negligible shrinking of the red blood cells, not exceeding 2.2%.

The main advantage is the short loading time of 15 min for $LiHCO_3$ compared with 3 hours for LiCl. Under these conditions saturating intracellular Li^+ concentrations can be obtained much more rapidly than with LiCl loading, thereby minimising alterations of the cell membranes. $LiHCO_3$ loading shortens the experimental time considerably and enables a greater number of samples to be screened from larger population cohorts.

Introduction

There have been several reports of increased sodium-lithium countertransport (SLC) in patients with essential hypertension (1, 2) and insulin-dependent diabetes with and without microalbuminuria (3–5). This sodium-lithium countertransport system exchanges Li^+ for Na^+ in an external Na^+-stimulated Li^+ efflux, and it shows saturation according to *Michaelis-Menten* kinetics (6). An elevated sodium-lithium countertransport activity in erythrocytes has been found in hypertensive subpopulations (1) and in patients with insulin-dependent diabetes mellitus complicated by renal disease (7, 8), but not in individuals with secondary cause of hypertension (9). In contrast, *Rutherford* et al. have reported (10) that the elevated sodium-lithium countertransport activity in patients with essential hypertension is attributable to a low K_m with no difference in V_{max}, as compared with normotensive subjects. Possibly, these discrepancies may partly be due to different assay methodologies or to selection bias.

Thus, the aim of this study was to validate the sodium-lithium countertransport measurements and to assess the influence of different loading media on the countertransport activity and its kinetics.

Materials and Methods

Preparation of red cell suspension

Venous blood from 8 non-fasted healthy volunteers (age: 25 ± 4 years, mean ± SD) was collected between 7.00–8.00 a.m., using Monovette® syringes (Sarstedt, Nümbrecht, Germany) containing EDTA, and centrifuged at 1500 *g*. The red cells were washed four

times with an isoosmotic washing buffer (75 mmol/l magnesium chloride/80 mmol/l sucrose/5 mmol/l glucose/10 mmol/l tris(hydroxymethyl)aminomethane-morpholino-propane sulphonic acid [Tris-MOPS], pH 7.55) at 20 °C. Osmolality was measured using a Knauer automatic semi-micro osmometer A300. Red cell suspensions were kept at room temperature and used within one hour.

Sodium-lithium countertransport activity – comparison of methods

Measurement of sodium-lithium countertransport after loading with lithium chloride

The method used was similar to that described by *Canessa* et al. (1). Four millilitres of washed red cells were incubated for three hours at 37 °C with shaking in 20 ml loading solution (150 mmol/l lithium chloride/10 mmol/l glucose/10 mmol/l Tris-MOPS, pH 7.55). The cells were washed five times with washing buffer to remove the extracellular lithium. An aliquot of the lithium-loaded cells was taken for determination of haematocrit, haemoglobin and the lithium content of the haemolysed cells by atomic absorption spectrometry (UNICAM Model Solaar 939). The lithium efflux was measured by incubating 1.8 ml of cell suspension (haematocrit 0.05–0.08) in a sodium-free medium and in a sodium medium as described in detail (11). After 0, 30, 60 and 90 minutes, incubation in either medium was stopped by cooling the tubes on ice for two minutes and subsequent centrifugation at 1500 *g* for four minutes at 4 °C. Lithium concentration in the supernatant was measured by atomic absorption spectrophotometry. Sodium-lithium countertransport activity was determined as the difference between the sodium-stimulated lithium efflux and the passive efflux in potassium chloride medium over time.

The intra-assay variation of our assay is 7.7% (n = 9) and the inter-assay variation is 15.3% (n = 4), where the inter-assay variation expresses both the variability of the test and the biological variability by measuring 4 samples during a four week period in five individuals. These assay variations are comparable to those reported in the literature (3, 5).

Measurement of sodium-lithium countertransport after loading with lithium bicarbonate

This method was carried out by the same procedure as described above except for the loading conditions. Instead of a lithium chloride solution, the erythrocytes were incubated in a lithium bicarbonate medium (150 mmol/l lithium bicarbonate/10 mmol/l glucose/10 mmol/l Tris-MOPS gassed with CO_2 until the pH value had adjusted to 7.55) according to *Ibsen* et al. (12) for 15 min. Thereafter the cells were washed five times and the efflux measurements were performed as mentioned above. Sodium-lithium countertransport values obtained with both methods were compared in 8 healthy subjects.

K_m and V_{max} of sodium-lithium countertransport

Erythrocytes were loaded with 150 mmol/l lithium chloride or 150 mmol/l lithium bicarbonate. The erythrocytes were then incubated in media containing 0, 50, 100, 150, 200 and 250 mmol/l NaCl, respectively. The media also contained 1 mmol/l $MgCl_2$/10 mmol/l glucose/0.1 mmol/l ouabain/10 mmol/l Tris-MOPS, pH 7.55. The solutions containing ≤ 100 mmol/l NaCl were made isotonic with potassium chloride according to *Ibsen* et al. (12) who found identical values for leak efflux in KCl medium and in media used in other laboratories such as cholinium chloride or magnesium chloride.

The *Michaelis* constant K_m and maximum velocity V_{max} were calculated by non-linear regression of the *Michaelis-Menten* equation $v = V_{max}\,[S]/(K_m + [S])$ using the *Marquardt-Levenberg* algorithm (see Statistical analysis). The kinetic constants K_m and V_{max} of sodium-lithium countertransport activity were determined in erythrocytes from 8 normal healthy subjects at the different Li^+ loadings with 150 mmol/l lithium chloride or 150 mmol/l lithium bicarbonate. The intra-assay variation for K_m was 13.5% (n = 16) and for V_{max} 6.3% (n = 16).

Cell volume measurements

Volume measurements were carried out with a cell counter CASY®1 (Schärfe System GmbH, Reutlingen, Germany). Erythrocyte suspensions were diluted to a final concentration of 30 × 10^6/l. The electronic pulse areas related to the particle passage through a 60 µm diameter capillary tube were analysed. Mean red cell volume was calculated from the size distribution curve between 4.0 and 6.0 µm cell diameter. Triplicate measurements were performed in 0.04 mol/l phosphate/saline buffer, pH 7.4 at room temperature.

Statistical analysis

Results with a normal distribution are expressed as mean ± SD. The normality was tested by the *Kolmogorov-Smirnov* test. The significance of differences between two groups was assayed using the *Student*'s t-test. P values (two-tailed) < 0.05 were considered statistically significant. Non-linear fitting of the *Michaelis-Menten* curve was performed by the computer software "SigmaPlot for Windows" (Jandel Scientific GmbH, Erkrath, Germany). Non-linear curve fit uses the *Marquardt-Levenberg* algorithm to find the coefficients K_m and V_{max}. This algorithm seeks the values of parameters that minimize the sum of the squared differences between the values of the observed and predicted values of the dependent variable.

Results

The relationship between sodium-lithium countertransport activity and external sodium concentration for the calculation of the *Michaelis* constant and maximum velocity are shown in figure 1. The kinetics of Li^+ efflux from erythrocytes of all healthy subjects studied, obtained with the two loading procedures, are demon-

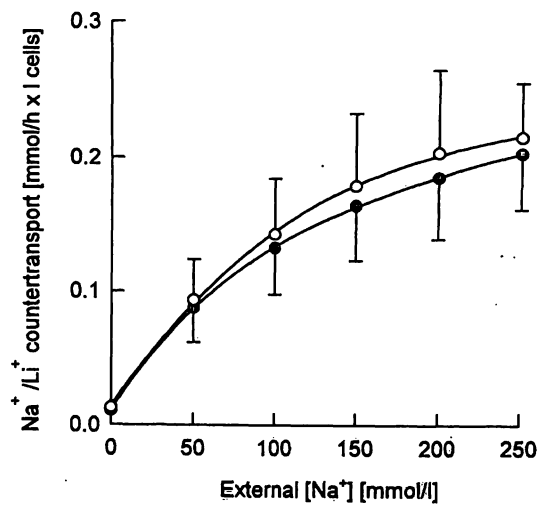

Fig. 1 Relationship between sodium-lithium countertransport activity and external sodium concentration of red blood cells after loading with 150 mmol/l LiCl (○) or 150 mmol/l $LiHCO_3$ (●). The kinetic experiments were run in duplicate, and each data point represents the mean ± SD of 8 individual blood samples.

strated in table 1. Sodium-lithium countertransport activity did not significantly differ between both loading procedures. Also, both the *Michaelis* constant and the maximum velocity were the same in erythrocytes loaded with lithium chloride or with lithium bicarbonate. Table 1 further shows that the intracellular Li^+ content significantly increased when lithium chloride was replaced by lithium bicarbonate. Osmolalities in the efflux media containing 200 and 250 mmol/l NaCl (tab. 2) were somewhat higher than in the other solutions. However, as shown in table 2, increase of the osmolality in those solutions resulted in a negligible shrinking of erythrocytes, not exceeding 2.2%.

Loading of the erythrocytes in $LiHCO_3$ was much faster than in LiCl medium, so that the intracellular Li^+ concentrations given in table 1 were attained within 15 min, compared with 180 min with the LiCl method (fig. 2). The increase of Li^+ concentration in red cells was nearly linear with time. Thus, saturation was not achieved under these conditions. The rates of both Na^+-induced Li^+ efflux (release into NaCl) and the spontaneous Na^+-independent release (release into KCl) were nearly equal after loading the erythrocytes in lithium bicarbonate or lithium chloride medium. The values for sodium-lithium countertransport activity, maximum velocity and *Michaelis* constant were not significantly influenced by the method used for their determination ($p > 0.1$), as demonstrated by the straight lines in figure 3.

Tab. 1 Influence on erythrocyte sodium-lithium countertransport kinetics of different counteranions in Li^+-loading medium. Eight normal healthy subjects were used for sodium-lithium countertransport measurements (mean ± SD).

	LiCl 150 mmol/l	$LiHCO_3$ 150 mmol/l
Sodium-lithium countertransport activity [mmol/h × l cells]	0.168 ± 0.062	0.165 ± 0.067
V_{max} [mmol/h × l cells]	0.336 ± 0.089	0.299 ± 0.070
K_m [mmol/l]	136 ± 27	127 ± 40
Li^+ content [mmol/l cells]	7.0 ± 1.4[1]	8.9 ± 1.5

[1] $p < 0.05$ vs. lithium bicarbonate

Tab. 2 Cell volume and osmolality of the erythrocytes in different media.

Media (mmol/l)	Cell volume[1] [µm³]	Change of mean volume[2] [µm³]	Osmolality [mmol/kg]
Loading media			
LiCl (150)	64.5	0.1	295
$LiHCO_3$ (150)	64.8	0.2	295
Efflux media			
KCl (150)	64.7	0.1	295
NaCl (50) + KCl (100)	64.8	0.2	295
NaCl (100) + KCl (50)	64.6	0	295
NaCl (150)	64.6	0	295
NaCl (200)	63.6	1.0	400
NaCl (250)	63.2	1.4	510

[1] Results obtained with two different samples
[2] Referred to erythrocyte volume in isotonic 150 mmol/l NaCl medium

Discussion

Discrepant findings have been reported in the literature with respect to the maximal activity and kinetic constants K_m and V_{max} of the erythrocyte sodium-lithium countertransport activity (9). This might be explained by either the metabolic status of the patients investigated, different assay methodologies or the use of an insufficiently high sodium concentration for their correct determination. *Canessa* et al. (9) have recently emphasized the importance of achieving sufficiently high intracellular Li^+ concentrations in order to ensure accurate measurement of sodium-lithium countertransport activity. *Duhm & Becker* (6) investigated the Na^+-dependent Li^+ transport across the human red cell membrane. Moreover, the mechanisms that lead to increased sodium-lithium countertransport activity in various disease conditions seem to be different, e. g. in hypertension the K_m is low, whereas in type I diabetes V_{max} is increased (10, 13). It is now clear that there are no significant differences in the maximal sodium-lithium countertransport

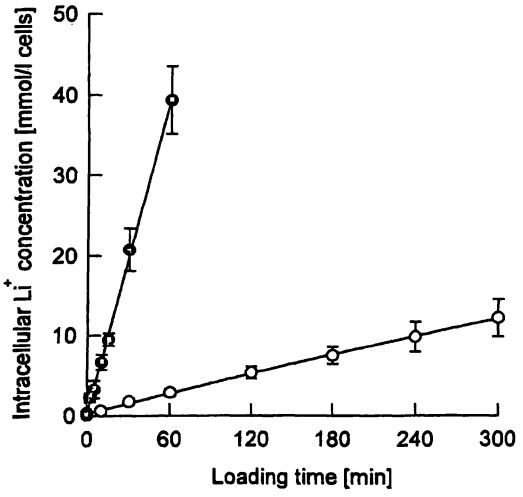

Fig. 2 Effect of the loading procedure on the time dependence of Li^+ concentration in human erythrocytes. Loading was performed with 150 mmol/l LiCl (○) and 150 mmol/l $LiHCO_3$ (●) at 37 °C. Each data point represents the mean ± SD of 5 individual blood samples.

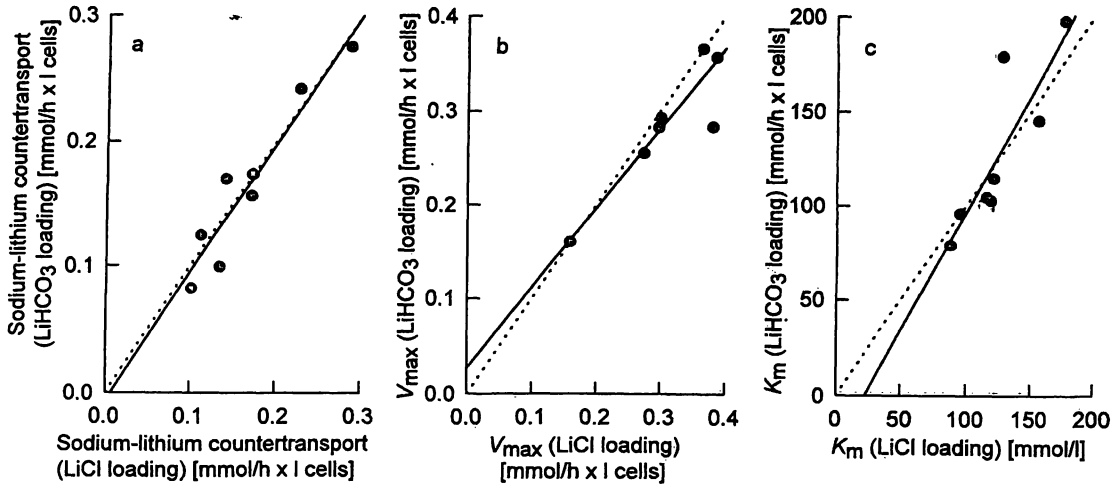

Fig. 3 Comparison of
a) sodium-lithium countertransport activity
b) maximum velocity (V_{max}) and
c) *Michaelis* constant (K_m)
of erythrocytes from 8 normal subjects after loading with either 150 mmol/l lithium bicarbonate (y-axis) or lithium chloride (x-axis). The equations of the regression lines (solid lines) are
a) y = 1.02x − 0.007; r = 0.948
b) y = 0.84x − 0.027; r = 0.915
c) y = 1.24x − 28.0; r = 0.859
The dotted lines represent the identity of both methods.

activity after loading the erythrocytes in either LiCl or $LiHCO_3$, indicating that intracellular Li^+ concentrations above 5 mmol/l allow precise Li^+ efflux measurements. It therefore seems unlikely that Li^+ loading with lithium bicarbonate would affect the sodium-lithium countertransport activity data reported in non-nephropathic and nephropathic diabetes by *Elving* et al. (3) or *Jensen* et al. (4). In previous studies, employing the well-characterised LiCl method, we found no significant differences in the erythrocyte sodium-lithium countertransport activity between subgroups of normoalbuminuric and microalbuminuric type I diabetic patients (11), although their activity was higher than in age-matched healthy control subjects. In this respect, our previous results compare well with those of *Elving* et al. (3) and *Jensen* et al. (4).

From the present results we may conclude that $LiHCO_3$ loading of erythrocytes results in a reliable and convenient method for measuring the sodium-lithium countertransport activity of erythrocytes. The main advantage is that under these conditions high intracellular Li^+ concentrations can be obtained, and much more rapidly than with LiCl loading, thereby minimising alterations of the cell membranes that may occur during lengthy incubations. Since the loading capacity of the erythrocytes is high in the presence of $LiHCO_3$, the loading time ought to be carefully controlled and strictly limited. $LiHCO_3$ loading considerably shortens the time needed for an experiment and enables a greater number of samples to be screened from larger population cohorts. With respect to the various mechanisms that induce changes of sodium-lithium countertransport activity in different disease states, it should be emphasised that determination of K_m and V_{max} requires carefully standardised loading conditions. The present loading procedure rapidly achieves Li^+ contents of more than 10 mmol/l red cells (see fig. 2) and circumvents the use of nystatin (9), a polyene antibiotic that possibly disrupts the internal electrolyte milieu and causes membrane damage (14, 15).

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